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Flow cytometry analysis of low/high DNA content (LNA/HNA) bacteria as bioindicator of water quality evaluation

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ABSTRACT

Anthropogenic activities such as agriculture, industry and climate change have been creating an enormous pressure on freshwater ecosystems leading to its degradation. Because the Water Framework Directive (WFD; main EU instrument for ecological water quality assessment) is costly and time-consuming, the main goal of this study was to use the scoring by flow cytometry (FCM) of bacterial communities with high DNA content (HNA) and low DNA content (LNA), a fast and easy methodology, as bioindicators. To portray this study, 3 sampling sites of Caima river with different levels of environmental impact (site 1 – no anthropogenic impacted site; site 2 – downstream wastewater treatment plant and site 3 – downstream a deactivated mine) were analysed along the 4 seasons of the year 2017 (winter, spring, summer and autumn). The ecological status of the sampling sites was accessed following the methodology described by WFD and biotic index results obtained for macroinvertebrate and periphyton communities, as well as the BOD_5, were compared with the bacterial community scored as HNA and LNA.

Bacteria community analysis showed high bacteria density at site 2 corresponding to high amounts of organic input by the wastewater treatment plant. Also, HNA bacteria were found to be in higher quantities at site 2, related to an increase of nutrients, while LNA bacteria were more prominent in river headwater, corresponding to an oligotrophic environment. Correlations between biological indices and bacteria community composition were very strong, showing that bacteria communities may serve as indicators of water quality assessment. Although this FCM technique provide good responses, further investigations are needed to confirm the feasibility of this method.

1. Introduction

Worldwide deterioration of water quality has been attributed to natural causes and anthropogenic activities, including hydrological features, climate change, precipitation, agricultural land use, sewage and runoff discharge. Conscientiously acting over this scenario, regulatory frameworks have been enforced worldwide to protect freshwater ecosystems. A major example in this context is the Water Framework Directive (WFD) within the European Union (EU). From 2000 onwards, all EU member states implemented the WFD as a basis for management decisions relying not only on physico-chemical monitoring, but also on biotic communities. Deterioration or improvement in ecological status are defined by the response of the biota, adding to changes in physical or chemical parameters (Hering et al., 2010). The biotic communities established as indicators for water quality assessment are currently benthic macroinvertebrates, periphyton, macrophytes and fish (EU 2000/60, 2000).

Bacteriological communities have for long been monitored in water quality assessment intended for human consumption (Directive 98/83/EC, 1998). Protective standards in this context are defined by the occurrence of selected indicator bacteria, mostly enterobacteria and faecal bacteria, which should be found in concentrations below a reference concentration expressed as the most probable number per volume (MPN/100 mL) or colony forming unit per volume (CFU/100 mL) (Boi et al., 2016; Liao et al., 2018). Recently, diverse features of aquatic bacterial communities were proposed as early indicators of changes occurring in riverine ecosystems (Boi et al., 2016; de Figueiredo et al., 2012, 2010; Servais et al., 2005). Bacterial abundance, activity and community composition largely vary in space and time, normally as a function of biotic and abiotic conditions seasonally fluctuating (Boi

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et al., 2016). Previous studies report an increase of bacterial abundance, biomass and metabolic rate correlating with the deterioration of freshwater trophic status (Boi et al., 2016; Sinsabaugh and Foreman, 2001; Stockner et al., 2000), namely linking to eutrophic and highly polluted environments (Boi et al., 2016; Delgiorgio and Scarborough, 1995; Yamaguchi et al., 1997).

In this way, we hypothesised that the bacterial community structure and composition analysis in riverine compartments (water column and sediment) could be informative for water quality evaluation purposes. Despite not being integrant part of the WFD, the sediment matrix (organic matter quantification and elutriate analysis) was considered in this study, given its overall contribution to the definition of water quality. In rivers, the water column stability is low, depending on the flow and turbulence, while sediments can accumulate organic and inorganic material bound to particles that may become bioavailable following sediment disturbance and resuspension (Burton, 2002). From a practical point of view, an efficient support of water quality assessment (especially if tuned to assist regulatory frameworks such as the WFD) requires the use of time- and cost-effective methodologies, this being the baseline rationale behind our focus on the potential of flow cytometry (FCM) to address riverine bacterial communities.

FCM combined with fluorescent stains has been used as a fast screening technique in drinking water quality assessment (Liu et al., 2016a; Prest et al., 2013, 2014) and more recently in rivers water quality assessment (Harry et al., 2016; Ibekwe et al., 2016; Liao et al., 2018), by acquiring information on total cell concentration and nucleic acid content of bacterial populations. Some fluorescent stains such as SYBR® Green I and SYTO 9, 13 bind preferentially to nucleic acids (Falcioni et al., 2006; Vives-Rego et al., 2000; Zipper et al., 2004), making it possible for FCM to measure bacterial concentrations. Moreover, the fluorescence intensity of such stains is directly related to the amount of nucleic acids present in the treated sample: fluorescence intensity recorded for one labelled bacterial cell should be directly related to its nucleic acid content, which is dependent on both the type of bacteria as well as its physiological state (Günther et al., 2008; Liu et al., 2016b; Prest et al., 2013). Based on the clear differences in fluorescence intensity and light side scattering (SSC) signals detected by FCM in combination with nucleic acid stains, bacteria have been broadly classified into two groups: low nucleic acid content (LNA) bacteria and high nucleic acid content (HNA) bacteria, thus creating a bacterial community "fingerprint" (De Roy et al., 2012; Liu et al., 2013; Romdhane et al., 2014). Thereby, FCM fingerprints provide information on the bacterial community characteristics and are a sensitive method for detecting small changes and shifts within the bacterial community (Van Nevel et al., 2017; Prest et al., 2014).

Thus, the aim of the present study was to gain a first insight on whether the bacteria community assessed using the FCM fast screening technique can be used as bioindicator of river water quality. Such a quality could benefit rapid, cost-efficient environmental bioassessment towards site prioritization for further in-depth analysis e.g. complying with the WFD, or also as a screening stage for integration within Environmental Risk Assessment frameworks. Caima River (Northern-Centre of Portugal) was selected as case study as it is a recipient of metal (effluents from a deactivated mine) and organic contamination (Nunes et al., 2003; Vidal et al., 2012), hence allowing to include both unimpacted and differentially impacted scenarios in the analysis. In this way, three sites were selected and seasonally assessed for water quality status *sensu* WFD (using periphyton and macroinvertebrate communities as biotic elements) and HNA and LNA bacteria content. This was expected to allow a direct view on whether bacteria endpoints correlate with traditional biotic metrics used for water quality assessment.

2. Material and methods

2.1. Study area and sampling

Caima River is a tributary of the Vouga River (Northern Center of Portugal). Caima River flows from its headwater at Serra da Freita (Arouca, Portugal; 900 m altitude) and flows to its mouth at Albergaria-a-Velha. Three sites were selected for this study. Site 1 is located near the river headwater (40°52′3.734″N, 8°16′22.199″W) and bears minimum human disturbance; site 2 is located downstream a wastewater treatment plant (WWTP) (40°48′21.258″N, 8°26′44.581″W), where domestic and industrial waste is likely dumped into the river; and site 3 is located nearby a deactivated mine (40°44′31.132″N, 8°27′16.222″W – Palhal mine-inactive since the 1920s), thus it is exposed to mine drainage, burdened with metals, especially lead (Vidal et al., 2012). Site 1 was affected by unforeseeable summer wildfires, followed by winter rains that promote the ashes runoff into the river course, before the first sampling took place, affecting headwater area in winter, with diffuse contamination by chemical elements in the aquatic ecosystem (Campos et al., 2016).

Each site was sampled seasonally, in January (winter), April (spring), June (summer) and September (autumn) of 2017, thus completing one year of sampling. Water temperature (°C), pH, conductivity (μS cm^{-1}) and dissolved oxygen (% and mg L^{-1}) were measured *in situ* using a multiparameter probe Aquaprobe AP-2000 (Aquaread®). Two liters of surface water were collected for further laboratorial physico-chemical characterization. Three replicate sterilized plastic containers (60 mL) were used to collect water samples for FCM analysis, from each sampling site. Sediment samples were taken from the top layer of the streambed into plastic airtight bags for elutriate production and consequent physico-chemical characterization and metal analysis. Both water and sediment samples were transported to the laboratory in the dark at 4 °C.

Benthic macroinvertebrates sampling was based on the proportional composition of microhabitats (Hering et al., 2003), by kick-sampling from small transects using a hand net (500 μm pore size; square frame 0.30 × 0.30 m) and a similar sampling effort (in time) across sites. Collected macroinvertebrates were stored in plastic containers, preserved with 80–90% of ethanol (Hauer and Resh, 1996).

Periphyton sampling followed EN 13946 (2003). Five small rocks were scraped with a hard toothbrush for removal of the top diatoms biofilm, representing a composed sample with an area of 100 cm^2. The samples were preserved with Lugol solution and transported to the laboratory at 4°C in the dark.

To additionally support the ecological classification of water, the hydromorphological elements (*sensu* WFD) were assessed based on the hydrology, morphology and continuity of the river. The methodology used was the River Habitat Survey (Raven et al., 1997, 1998, 2002) and the hydromorphological quality was based on two indices: Habitat Modification Score (HMS) and Habitat Quality Assessment (HQA). The HMS evaluates the artificiality of the physical structure of the channel that means the impact of man-made structures in the river. The HQA integrates the bed characteristics and river corridor conditions that contributes to the success of the biological communities. In both indices, the limits and classes of quality were based in national guidelines (INAG, 2009). In short, the River Habitat Survey methodology was based on the evidence of flow regime, platform pattern, variability of cross-section by width and depth, lateral and longitudinal extension of vegetation and river corridor structure, erosional and depositional features, bed substrate and bed configuration, structures with impact on longitudinal continuity, artificial elements, aquatic vegetation, structure of bank vegetation and land use. The assessment was carried out in 10 spot-checks along 500 m of the river channel and with continuously observation along the river (sweep-up), as well as 50 m on each side of the channel for each site. When necessary, the field observations were completed with information retrieved from Google Earth®. The

software Rapid 3.0 (Davy-Bower et al., 2017) was used to calculate both indices.

2.2. Sediment treatment for further analysis

Immediately upon arrival to the laboratory, a subsample of sediment was separated and sorted to remove debris before organic matter content determination (see Section 2.3). The remaining was immediately used for elutriate preparation undergoing further analyses (see Section 2.3). Sediment was mixed 1:4 (v/v) with ultrapure water and shaken in an orbital shaker at 200 rpm during 2 h at 20 °C, then left to deposit overnight. The supernatant was centrifuged at 2500 × g for 15 min at 4°C and transferred to a clean erlenmeyer following procedures formerly described by Nebeker et al. (1984) and Ankley et al. (1991).

2.3. Physico-chemical quantifications

For the determination of organic matter content of sediments, the sorted sediment subsample was oven-dried at 70°C for 24 h before incineration in a muffle furnace at 450°C for 6 h (Kristensen and Andersen, 1987). Conductivity, dissolved oxygen and pH were recorded in readily prepared elutriates (see Section 2.2) using a multiparameter water quality probe (Aquaprobe AP-2000 (Aquaread®)).

Biochemical oxygen demand (BOD_5), dissolved organic carbon (DOC), turbidity, ammonia, total phosphorus (TP), total nitrogen (TN) and total suspended solids (TSS) were quantified in water and elutriate samples, according to APHA (1995). Both water and elutriate samples were acidified to pH < 2 with nitric acid PA 65% and analyzed by Atomic Absorption Spectrometry (AAS) for metal (Al, Mn, Fe, Cu, Zn, Cd, Ba, Pb, As and Cr) and total S quantification.

2.4. Macroinvertebrate and periphyton community analysis

Preserved macroinvertebrates were counted and identified to the lowest practicable taxonomic level, in this case the family level, using different identification keys (Edington and Hildrew, 2005; Elliott and Humpesch, 2010; Hynes, 1993; Pawley et al., 2011; Sundermann et al., 2007; Tachet et al., 2000; Wallace et al., 2003).

Ecological Quality Ratios (EQRs) were derived from the multimetric index $IPtI_N$ (North Invertebrate Portuguese Index (Eq. (1)); equivalent to ICM 7/STAR; Munne and Prat, 2009).

$$IPtI_N = N°\text{Taxa} \times 0.25 + \text{EPT} \times 0.15 + \text{Evenness} \times 0.1 + (\text{IASPT} - 2) \times 0.3 + \log(\text{Sel. ETD} + 1) \times 0.2 \quad (1)$$

where EPT is the number of Ephemeroptera, Trichoptera and Plecoptera taxa, IBMWP is the sum of pre-defined tolerance (to pollution) scores for each taxon (Alba-Tercedor and Sánchez-Ortega, 1988), IASPT is the average score taxon, derived from IBMWP, sel. ETD + 1 is the sum of family abundances of Heptageniidae, Ephemeridae, Brachycentridae, Goeridae, Odontoceridae, Limnephilidae, Polycentropodidae, Athericidae, Dixidae, Dolichopodidae, Empididae and Stratiomyidae and Evenness is calculated from equitability (Pielou's J') (INAG, 2009).

Reference values for all metrics were obtained from national guidance documents (INAG, 2009) considering Northern rivers of medium/large dimension, categorized by the following intervals: high, if EQR > 0.87; good, if 0.87 > EQR > 0.65; Moderate, if 0.65 > EQR > 0.44; Poor, if 0.44 > EQR > 0.22; and Bad, if EQR < 0.22.

Preserved periphyton samples were oxidized using the HNO_3 (67%, PA Merck) and (K_2 C_{12} O_7 Panreac, 99.5% purity) method until complete removal of organic material (EN 13946, 2003). A small amount of each sample was used to prepare permanent slides with Naphrax (Brunel Microscopes Ltd, UK). Diatom counting followed standard procedures EN (2004) with a minimum of 400 valves per sample counted and identified to the species or infra-specific level under a light microscope (Olympus CX 31) equipped with 100x immersion objective of 1.25NA, mostly with the support of standard floras (Krammer and Lange-Bertalot, 1991a,b, 1988, 1986; Werum and Lange-Bertalot, 2004). The diatom IPS index (Polluosensibility Index (Eq. (2)); Cemagref (1982)) was calculated with software OMNIDIA (v 6.0 – Lecointe et al, 1993).

$$\text{IPS} = \frac{\left(\sum_{j=1}^{n} a_j s_j v_j\right)}{\left(\sum_{j=1}^{n} a_j s_j\right)} \quad (2)$$

where a_j stands for the abundance of species j; s_j stands for the sensitivity of the species j towards the disturbance degree; v_j stands for the species indicator value.

2.5. Bacterial community analysis by FCM

FCM analysis were performed in water samples and elutriates using a commercial kit (Bacteria Counting Kit, Molecular Probes™, Invitrogen) for accurate enumeration of bacteria. The manufacturer's protocol was strictly followed for further analysis of the samples in an Attune® Acoustic Focusing Cytometer (TermoFisher Scientific) equipped with a 488 nm laser. In short, collected samples were added SYTO BC and the mixture was incubated at 37 °C during at least 5 min; then, the microsphere standard suspension was added to the stained cell preparation and this sample underwent FCM reading. The SYTO BC was excited at 488 nm and fluorescence measured with 530/30 bandpass filter (BL1). Light forward scatter (FSC) and SSC were also recorded. For statistical significance, at least 10^5 cells were analyzed in each sample and bacteria populations were selected based on BL1 and FSC profiles using the FlowJo software (Tree Star Inc., Ashland, OR, USA). In the BL1 vs SSC cytogram, a polygonal region was defined to include only bacteria populations and the concentration of cells in this region was recorded. A marker for separation of LNA (low nucleic acid) and HNA (high nucleic acid) was defined in the BL1 vs SSC cytogram and the concentration of cells in each subpopulation was also recorded.

2.6. Integrated data analysis

$IPtI_N$ and IPS, as integrated biotic indices denoting water quality *sensu* WFD were correlated (non-parametric Spearman correlation provided n = 3 sites) with bacteria total cellular density, high DNA content and low DNA content bacteria, under the null hypothesis that there is no monotonic relationship between variables (Quinn and Keough, 2002). BOD_5 was also correlated with the bacteria variables given the evidence by Sharuddin et al. (2018) correlating it with total density of bacteria and HNA and LNA. BOD_5 indirectly translates the water samples organic load by accounting the amount of dissolved oxygen used by microorganisms in the biological processes of metabolizing organic matter, in water, and therefore the more organic matter present, the greater the BOD_5 value. Correlation analysis was performed in Statistical Software MINITAB Inc. version 16 following previous rank transformation of the data.

3. Results and discussion

3.1. Water quality assessment sensu WFD

The basic physical and chemical parameters for water and sediment elutriates are shown in Tables S1 and S2, respectively. Caima River headwaters recorded invariably the highest pH values (maximum of 9.14 in winter) and in site 3, downstream a deactivated mine pH around 7 was always recorded, regardless the season. No extreme records were taken regarding conductivity (cond) and dissolved oxygen (O_2); these

were, in general, constant through the year, ranging within 1–150 μS cm^{-1} above 70%, respectively. In general, ammonium (NH_4; 0.08–3.66 mg L^{-1}), ammonia (NH_3; 0.07–3.46 mg L^{-1}), total nitrogen (TN; 0–1.52 mg L^{-1}), phosphate (PO_4; 0–0.74 mg L^{-1}), total phosphorus (TP; 0–0.24 mg L^{-1}) and nitrate (NO_3; 0–6.73 mg L^{-1}) recorded the highest values at site 2 in all seasons. Dissolved organic carbon (DOC) recorded the highest values in spring and autumn. Biochemical oxygen demand (BOD_5) fluctuated between 0.31 and 6.25 mg L^{-1}, being higher in winter for all sampling sites. BOD_5 values were within the data range between 1 (detection limit) and 20 mg L^{-1} obtained in another study (Miltner, 2018) where they studied Ohio river for the last 10–15 years, a densely populated area subjected to waste water discharges, intensive agriculture and livestock farming. Meanwhile, no BOD_5 values above 6.25 mg L^{-1} were obtained showing that Caima River was in lower eutrophication level. Organic matter (OM) in sediments was always higher at site 1 and lower at site 3 throughout the seasons. The OM values recorded were also comparable with values obtained for a semi-natural drainage catchment in a nearby area (Pereira et al., 2017). The physico-chemical parameters measurement must fulfill established threshold values, depending on the river typology, for reaching good ecological status. Parameter values such as dissolved O_2 concentration ≥5 mg/L and ranging within 60%–120%, BOD_5 below 6 mg/L, pH within 6–9, NH_4 below 1 mg/L, NO_3 below 25 mg/L and TP below 0.10 mg/L define good quality status in medium-large dimension rivers of northern Portugal. These criteria were not met for all sampling sites in some seasons (see Table S1 for the raw data), resulting in a lower-quality classification (Table 1). Site 2 was classified as moderate along the four seasons, with high values of TP in all seasons except autumn, and high levels of NH_4 in winter and autumn. Also, in winter, site 3 and site 1 obtained moderate classification due to an elevated value of BOD_5 (6.25) and a high value of pH (9.14), respectively (Table 1). Regarding the specific pollutants, chemical status and hydromorphological quality elements, all sampled sites were qualified as good (Table 1).

Specific pollutants and all trace elements quantified in water samples were below the safety thresholds by WFD regulation (WFD; Directive 2000/60/EC- Establishing a Framework for Community Action in the Field of Water Policy). This suggests that trace elements burden at the monitored sites is not environmentally hazardous. Trace element concentrations were always higher in elutriates (Table S3) through the seasons, except for sulfur, which was quantified at higher concentrations in water (Table S4).

Elements such as zinc (Zn; 0.05 mg L^{-1}), cadmium (Cd; 0.001 mg L^{-1}) and barium (Ba; 0.01 mg L^{-1}) were found in very low concentrations throughout the year, both in water and elutriate samples. Concentrations of manganese (Mn; 0.002–0.57 mg L^{-1}), lead (Pb; 0.003–0.009 mg L^{-1}) and total Sulphur (S; 0.13–2.26 mg L^{-1}) were found to vary among seasons, although it seems that in spring the values are lower compared to the other seasons. Remarkably, arsenic (As; 0.003–0.004 mg L^{-1}), aluminum (Al; 0.019–3.68 mg L^{-1}) and copper (Cu; 0.002–0.009 mg L^{-1}) recorded high concentrations in elutriates at site 1 in winter, generally decreasing gradually along the seasons. Also, the maximum values of Al and Cu, in water samples were found at river headwaters, in spring. Mn, Fe and As in water and Cu, in sediments, had their maximum values at site 2, in autumn. Fe and Cr recorded the maximum values in site 1, in summer and spring, respectively. These results are in line with literature that found metals such as S, Al, Cu, As, Cr and Fe (Campos et al., 2012; Silva et al., 2015) and nutrients, especially phosphate, nitrate and ammonium (Earl and Blinn, 2003; Hosseini et al., 2017; Spencer et al., 2003), attached to ashes and subsequently transported to different environmental compartments. Ash transport into waterways may indeed explain the higher concentrations of these metals and nutrients found mainly in elutriate and water samples (derived from sediments where incoming material may accumulate), as well as the high values of pH recorded in winter at river headwater (Earl and Blinn, 2003). High OM recorded in sediments at site 1 might also be related with the limited amount of sediments available on site 1; this required the complementary sediment collection slightly downstream in a low-flow area were sediment deposition occurs. It is likely that this caused the overrated nutrient and metals recorded in site 1.

Regarding the biological communities, $IPtI_N$ (macroinvertebrate community) and IPS (periphyton community) indices, as well as derived EQR ratios are summarized in Table 1. At the river headwater, EQR values were always higher than in the other sites through seasons, considering the macroinvertebrate community. Here, *taxa* known as sensitive to organic pollution (Chessman and McEvoy, 1997; Lydy et al., 2000) were found in higher quantity, pointing site 1 as a reference and showing no evidence of negative effects in the macroinvertebrate community by ash load. Site 2 was classified as moderate, reaching poor quality in summer, which possibly relates to high temperatures and low precipitation, resulting in lower river flow and consequently lower dilution of WWTP incomes that may have negatively affect macroinvertebrate communities. Site 3 quality status was found to be good through the 4 seasons. As far as periphyton communities are concerned, water quality was good for all sampling sites in winter and

Table 1
Detailed perspective on the integration of different element's classification towards final ecological status classification of the studied sites *sensu* WFD. The actual values of biological indices (IPS and $IPtI_N$) originating different classification classes (INAG, 2009) are given within brackets.

		Biological Quality		HydromorphologicQuality	Specific Pollutants	Physico-chemical Quality	ECOLOGICAL STATUS	CHEMICAL STATUS	FINAL ECOLOGICAL STATUS
		Periphyton (IPS)	Macroinvertebrate ($IPtI_N$)						
Winter	Site 1	Good (17.2)	High (0.983)	Good	Good	Moderate	Moderate	Good	Moderate
	Site 2	Good (18.4)	Moderate (0.499)	Good	Good	Moderate	Moderate	Good	Moderate
	Site 3	Good (18.1)	Good (0.787)	Good	Good	Moderate	Moderate	Good	Moderate
Spring	Site 1	Good (15.8)	Good (0.830)	Good	Good	Good	Good	Good	Good
	Site 2	Good (16.9)	Moderate (0.557)	Good	Good	Moderate	Moderate	Good	Moderate
	Site 3	Good (18.8)	Good (0.784)	Good	Good	Good	Good	Good	Good
Summer	Site 1	Good (16.4)	High (1.065)	Good	Good	Good	Good	Good	Good
	Site 2	High (18.9)	Poor (0.349)	Good	Good	Moderate	Poor	Good	Poor
	Site 3	Moderate (12.6)	Good (0.809)	Good	Good	Good	Moderate	Good	Moderate
Autumn	Site 1	High (19.9)	High (0.913)	Good	Good	Good	Good	Good	Good
	Site 2	Moderate (10.1)	Moderate (0.540)	Good	Good	Moderate	Moderate	Good	Moderate
	Site 3	Moderate (11.8)	Good (0.748)	Good	Good	Moderate	Moderate	Good	Moderate

spring, as well as in summer at site 1. High ecological status was recorded at site 1, in autumn, and unexpectedly at site 2, in summer. The disagreement between the two biological communities regarding water quality status discrimination is noticeable. Similar inconsistencies have been reported for ecosystems affected by ash inflows (Earl and Blinn, 2003).

All evaluated elements (biological communities, specific pollutants, hydromorphological quality elements, physico-chemical parameters and chemical quantification) were integrated to reach the final classification of ecological status of waterbodies (Table 1). The element resulting in the worst classification will be determinant following the rule of one out – all out (EU Directive 2008/105). Generally, the biological communities were the determinant element, except for site 1 and 3, in winter, where the ecological status were determined by the physico-chemical measurements. This justified our option to favor biological elements in correlation analyses over the remaining elements.

3.2. Bacteria analysis using FCM

There was always a higher concentration of bacteria (HNA and LNA) in elutriates than in water samples. The highest densities of HNA bacteria were recorded at site 2, mostly in the warmer seasons, while the opposite was recorded for LNA bacteria, reaching higher densities at site 1 in winter, for both water and elutriate samples (Table S5).

In general, LNA bacteria in water samples recorded the highest density at site 1 and lowest density at site 2, with an intermediate situation at site 3, except for summer, where site 3 reach the highest density.

In elutriate samples, the same pattern described for water samples was maintained, except for winter and spring, in which LNA bacteria density reach the lowest value in site 3 and site 1, respectively. In general, higher densities of HNA bacteria for both water samples and elutriate were recorded at site 2 (see Table S5). Disturbances like WWTP are known to promote favorable conditions for bacteria proliferation (Chróst et al., 2009; Liao et al., 2018). Abzazou et al. (2015) found that water samples with high organic loading rates had twice as much bacteria concentration than other samples. Accordingly, site 2 showed higher bacteria density compared to the other sites (Table S5). Site 1 was the least anthropogenically impacted site and recorded the highest ratio LNA:HNA bacteria density.

Additionally, Liao et al. (2018) and Wang et al. (2009) found that microbial community composition can respond to physicochemical characteristics of water affected by wastewater discharge like electrical conductivity, total concentration of nitrogen and phosphorous, as also evidenced in our results (Tables S1 and S2). Site 3 reflects an intermediate scenario (Fig. 1 and Table S5) between the other two sampling sites, with higher LNA bacteria density than site 2, but smaller than the river headwater (site 1), more clearly evidenced in water samples. Although no relationship was found in literature between metal contamination and river microbial communities, this intermediate position of site 3 results indicate that microbial communities can be impacted by the metal burden present in site 3.

In general, LNA bacteria ratio was higher in water samples (Fig. 1 and Table S5) with minimum contamination (site 1), in agreement to what has been mentioned by other authors (Salcher et al., 2011; Servais et al., 2003; Sharuddin et al., 2018). LNA bacteria were previously interpreted as not being active or even dead, but thereafter, several authors (Bouvier et al., 2007; Longnecker et al., 2005; Prest et al., 2013; Salcher et al., 2011; Servais et al., 2003; Wang et al., 2009) have shown that they are metabolically active cells which could survive in oligotrophic conditions due to their high affinity and binding-protein dependent uptake system. This suggests that LNA bacteria play a role as an active member of the community, especially in nutrient-limited environments. On the other hand, HNA bacteria are considered to be more dynamic and sensitive to environmental changes than LNA bacteria, being the former highly related with increased levels of nutrients

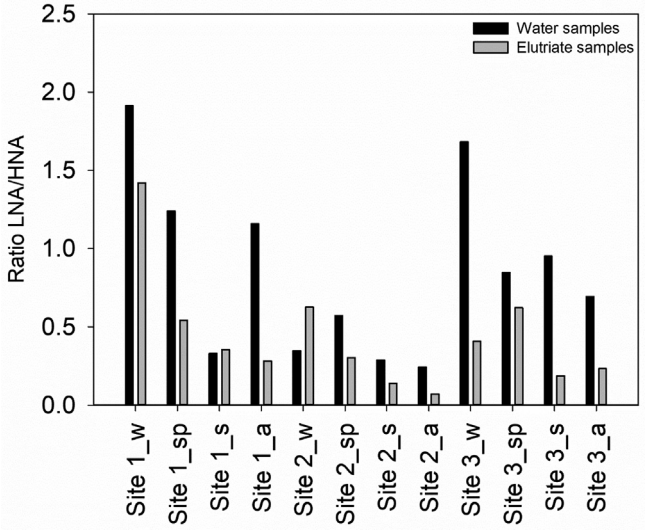

Fig. 1. LNA/HNA (cell ml^{-1}) ratios obtained for water and elutriate samples of the three sampling sites (1, 2 and 3) in four seasons (w-winter, sp-spring, s-summer; a-autumn).

especially phosphates, nitrates, conductivity and manganese (Wang et al., 2009), which is in complete agreement with our results (Figs S1, S2 and Table S5). Consistently, Sharuddin et al. (2018) pointed out HNA bacteria as bioindicators for screening anthropogenic organic inputs in river waters.

In this context, it is worth noting that a given bacterium can be read as LNA or HNA, depending on environmental conditions. This was recently clarified as conflicting classifications were used by different authors: Wang et al. (2009) classified *Polynucleobacter* as LNA cells and Martinez-Garcia et al. (2012) consider it as HNA bacteria cells. Sharuddin et al. (2018) addressed this inconsistency in classification and argue that LNA bacteria should be dormant under oligotrophic conditions, but then switch to an active condition (translating into increased DNA replication, thus DNA content) following organic load of the water. This leads to a HNA reading on a community that before read as LNA. In practice, such a feature enhances the bioindicator value of HNA/LNA ratios since they sensitively respond to fluctuations in environmental conditions rather than they are only dependent on the community *taxon* composition.

FCM has considerable potential application for bacterial water quality monitoring and early warning detection of changes in the bacteriological communities; it is officially accepted in Switzerland (SLMB, 2012) as a standard method for total bacteria cell counts in drinking water. Fluorescent fingerprinting is fast, simple and easy to standardize using fixed gating positions for LNA and HNA bacteria, allowing an easy understanding of the shifting of nucleic acid content of bacterial communities exposed to contamination. Prest et al. (2013) showed that, when reproducible staining and analysis procedures are used, FCM-based analysis is highly repeatable and allow discrimination between water samples from different origins; likewise, the FCM methodology herein allowed to discriminate among samples taken from riverine sites under different pressures (Fig. 1, Figs. S1 and S2). Certainly there are other techniques allowing the analysis of bacterial communities in aquatic matrices. One of such, perhaps that currently under the spotlight, is DNA metabarcoding. Its resolution is by far higher than that allowed by FCM-based methodologies, allowing taxonomic identification and corresponding functional analyses for example (Salis et al., 2017). However, DNA metabarcoding is also more complex and and demanding than FCM since it is costlier in terms of time, budget and expertise (mostly bioinformatics) requirements. In this way, FCM-based analysis fits screening stages of environmental assessment applicable e.g. in the prioritization of putatively impacted sites while DNA-

Table 2
Spearman correlation coefficients found while correlating biologicals indices ($IPtI_N$ and IPS) and BOD5 with bacteria parameters, regarding water and elutriate samples collected seasonally.

		Water			Elutriate		
	FCM endpoints	$IPtI_N$	IPS	BOD_5	$IPtI_N$	IPS	BOD_5
Winter	HNA	−1	1	0	−1	1	−0.5
	LNA	−0.887	0.5	−0.866	0.5	−0.5	1
Spring	HNA	−1	0.5	−1	−0.5	−0.5	0.5
	LNA	−0.5	−0.5	−0.5	−0.5	−0.5	0.5
Summer	HNA	−1	0.5	1	−0.5	1	1
	LNA	−0.5	−0.5	0.5	0.5	0.5	0.5
Autumn	HNA	−1	−1	1	−0.5	−0.5	1
	LNA	−1	−1	1	−0.5	−0.5	1

barcoding and relatives are advanced tools available for higher-tier bioassessment.

3.3. Correlating standard water quality assessment with bacteria quantification

Spearman correlation analysis was run between $IPtI_N$, IPS index, BOD_5 and bacteria community composition endpoints (high DNA content and low DNA content) for each sampling site considering both matrices (water and elutriate) in the four sampling seasons (Table 2).

All the correlations for each season retrieved absolute Spearman coefficients ranging within 0.5 (moderate) and 1 (very strong). Moreover, in water samples, there was a higher number of stronger correlations (Table 2). For instance, HNA bacteria showed a strong negative correlation (−1) with $IPtI_N$ index in all seasons, for water samples. Also, it is noticeable that $IPtI_N$ tends to correlate more strongly with bacteria parameters than IPS.

The BOD_5 parameter showed to be highly correlated with HNA bacteria in summer and autumn, for both water and sediment samples. Negative relationships between biotic multimetric indices were the most predominant both for water and elutriate samples, while BOD_5 tends to positively correlate with bacteria parameters.

There were strong correlations between bacterial communities and biological indices, showing that bacterial community composition may serve as indicators of anthropogenic inputs in river waters. Likewise, Liao et al. (2018) found a straightforward relationship between microbial community and water quality indices (natural organic matter metabolic index). Sharuddin et al. (2018) found high correlation between the total bacteria density and BOD_5 and Total Organic Carbon (TOC) similar to the strong correlation obtained in the present study.

4. Conclusions

This work intends to contribute to elucidate on the relationship between the microbial communities in rivers and its water quality assessment *sensu* WFD. Previous studies as Liao et al. (2018) and Sharuddin et al. (2018) also deal with the relationship between water quality and microbiological communities in rivers. In general, our results showed a strong correlation between the bacteria community data quantification by FCM, for water and elutriate samples, and biotic indices used in water quality evaluation, through the four seasons studied. The river headwater showed the largest proportion of LNA bacteria, in winter and spring, and the WWTP, nearby site 2, apparently increase largely the microbiological community specifically the HNA fraction responding to nutrient increase (mostly TN and TP). Nevertheless, this was a case study representing one river that must be scaled-up to comprise other river typologies and better appraise the value of these indicators. Different contamination scenarios (e.g. pesticides; complex mixtures) should be used to validate if the pattern of response generally applies. FCM applied to water quality assessment would be a fast, precise and easy tool added to the toolbox of WFD complementary approaches for screening and prioritization of sampling sites, requiring further detailed analysis of ecological status using WFD.

Competing interests

None

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2019.03.033.

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